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DETERMINATION OF NEURONAL CELL FATE: LESSONS FROM THE R7 NEURON OF *DROSOPHILA*

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INTRODUCTION

How is the remarkable cellular diversity in the nervous system established during development? Do cells assume specific fates as a consequence of intrinsic factors, or are they acquired through cellular interactions? Cell lineage studies provide a way to distinguish between these two extreme mechanistic alternatives. For instance, lineage studies in the vertebrate central nervous system (CNS) reveal that at late stages of development pluripotent cells exist within the neuroepithelium that give rise to different neuronal and glial cell types (e.g. Turner & Cepko 1987). Qualitatively similar results were obtained from genetic mosaic studies carried out some 20 years ago, which showed that there were no strict lineage relationships between different classes of neurons and support cells in the compound eye of *Drosophila melanogaster* (Ready et al 1976, Lawrence & Green 1979). In the absence of lineage, a prominent role for cellular interactions in regulating the development of both the fly eye and the vertebrate CNS has been proposed. In recent years considerable progress has been made in understanding the mechanisms by

which pattern formation and cell fate are regulated in the developing compound eye of *D. melanogaster*. (For general reviews of *D. melanogaster* eye development, see Tomlinson 1988, Ready 1989, Cagan & Zipursky 1992). We anticipate that similar mechanisms will be shown to play important roles in regulating cell fate determination in the vertebrate CNS.

In this review we focus on the development of one particular cell type in the compound eye, the R7 photoreceptor neuron. Through a multifaceted approach utilizing tools of cell biology, genetics, and biochemistry, important insights have been gained into the molecular strategies of cell fate determination. We discuss in detail the molecular nature of the cellular interactions leading to the induction of the R7 cell, the mechanisms that restrict induction to a subclass of cells in the developing eye, and the mechanisms by which the signal is transduced in the responding cells.

Several features of compound eye development make it a favorable system for molecular and genetic studies. First, the compound eye has a relatively simple modular structure. It comprises some 800 identical eyes called ommatidia (Figure 1), each containing 8 photoreceptor neurons (R cells), which minimally fall into 5 classes. There is one R7 cell within each ommatidium; it is a distinct neuronal cell type defined by its position, morphology, action spectrum, and synaptic connections. In addition to the R cells, each ommatidium contains nonneuronal support cells, including the cone cells, which secrete the simple positive lens capping each ommatidium, and the pigment cells, which ensheath the R cells, thereby optically isolating them from R cells in adjacent ommatidia. Second, the cellular dynamics of pattern formation have been described with single cell resolution (Ready et al 1976, Tomlinson 1985, Tomlinson & Ready 1987b, Cagan & Ready 1989, Wolff & Ready 1991). This description has provided a critical foundation of knowledge for interpreting developmental defects in mutants. Third, the eye is a dispensible structure; it is straightforward to identify mutations affecting the developing eye. As we shall see, the reiterative nature of the compound eye has proven to be an important feature in the design of highly successful genetic screens for interacting genes defining the R7 pathway. Recently developed genetic gadgetry facilitates the identification of mutations in genes that are essential for development to proceed through embryogenesis and that are also required for the development of postembryonic structures, such as the compound eye (Xu & Rubin 1993). Hence, in principle, all the genes necessary for compound eye development can be isolated. And finally, it is straightforward to clone genes from *D. melanogaster* and to reintroduce them into the germline under either their own promoter or one that drives their expression in different subsets of cells in the developing eye (reviewed in Rubin 1988).

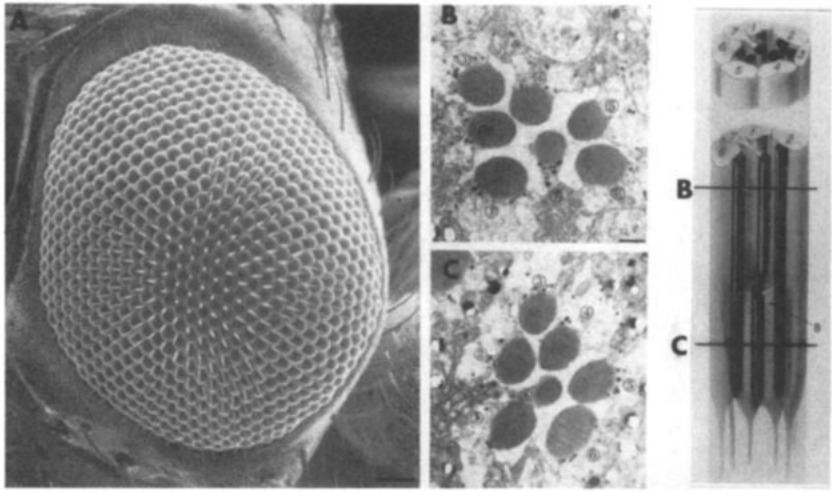

Figure 1 The structure of the compound eye of *Drosophila melanogaster*. (*A*) Scanning electron micrograph of the compound eye. The compound eye is a reiterated pattern of hexagonally arranged units called ommatidia. Each ommatidium is comprised of an invariant number of cells including eight photoreceptor neurons (R cells) and additional nonneuronal cells. (*B*) and (*C*) Transmission electron micrographs of sections in the distal and proximal regions of the ommatidium, respectively. The large, electron-dense structures are the rhabdomeres (Rh), the photosensitive organelles of the R cells. The panel on the right is a schematic representation of the cluster of R cells in an adult ommatidium. Scale bar is 10 μm in *A* and 1 μm in *B* and *C*. Panels *B* and *C* and the schematic representations are from Reinke & Zipursky (1988); reprinted by permission of publisher.

INDUCTION IN THE COMPOUND EYE

Assembly of ommatidia begins in the third larval instar in a columnar epithelium called the eye imaginal disc (Figure 2). Ommatidial development does not occur synchronously throughout the disc, but instead begins at the posterior edge and progresses anteriorly. Eye discs removed from larvae just prior to pupariation show a smoothly graded series of ommatidia at different stages of development, covering just over half of the disc (Ready et al 1976; see Figure 2B). Examination of individual cells in the forming ommatidia has shown that the photoreceptors differentiate in a fixed sequence, beginning with the central R8 photoreceptor and proceeding pairwise with R2 and R5, R3 and R4, R1 and R6, and finally R7 (Tomlinson & Ready 1987b; see Figure 2B). Although photoreceptor differentiation occurs in this fixed sequence, genetic mosaic analysis has failed to detect any role for cell-lineage relation-

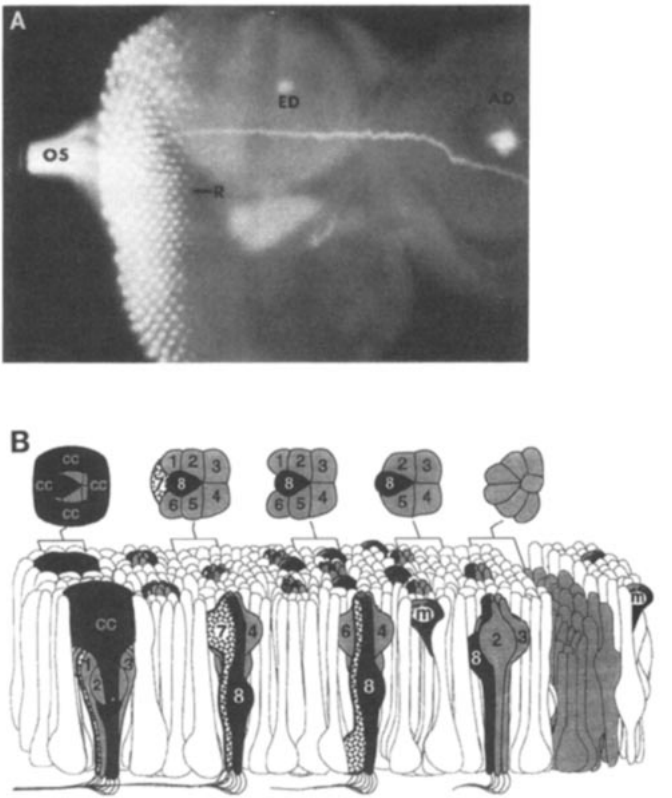

Figure 2 The development of the compound eye. The primordium of the compound eye, the eye imaginal disc, is set aside in early development as an invagination of the embryonic ectoderm. These cells proliferate during early larval development. Pattern formation and differentiation commence in the third and final stage of larval development. The retinal primordium is referred to as the eye imaginal disc.

Panel *A* shows a differentiating eye primordium, referred to as the eye imaginal disc, stained with a monoclonal antibody to chaoptin, a membraneglycoprotein expressed on the surface of developing photoreceptor neurons. Pattern formation in the eye commences in the posterior region and progresses anteriorly as a wave. (Posterior is to the left.) The leading edge of this wave is called the morphogenetic furrow. The following structures are shown: *AD*, antennal disc; *ED*, eye disc; *OS*, optic stalk; *R*, R cell cluster. Scale bar, 50 μm.

Panel *B* is a schematic representation of development in the eye imaginal disc. Because development proceeds as a wave across the disc, each disc represents a series of older steps in ommatidial assembly as one proceeds from the morphogenetic furrow (*b*) towards the posterior (*left*). (*a*) Anterior to the furrow, cells are actively dividing and unpatterned. Although there are no overt signs of cellular differentiation or pattern formation in this region of the disc, a few molecular markers are expressed in this region. (*b*) As cells enter the morphogenetic furrow (MF) they form an array of clusters containing 10–15 cells. It is thought that the MF reflects a series

ships in determining cell fates in the developing fly retina (Ready et al 1976, Lawrence & Green 1979). These genetic and morphological observations led to the proposal that retinal cell fate is governed by the specific combination of signals received by a cell from its immediate neighbors (Tomlinson & Ready 1987b). Such a mechanism would likely require the precise temporal and spatial regulation of the genes directly involved in generating, receiving, and interpreting these signals.

Recruitment of the R7 Cell

The R7 photoreceptor is the last of the eight photoreceptors to differentiate. Mutation of the *sevenless* (*sev*, Tomlinson & Ready 1986, 1987a) or *bride of sevenless* (*boss;* Reinke & Zipursky 1988) genes leads to a transformation of the R7 precursor cell—the cell that finds itself in the pocket created by the differentiating R8, R1, and R6 cells—into a nonneuronal, lens-secreting cone cell (Figures 2 and 3). Thus, the R7 precursor cell appears to face a simple choice between two alternative cell fates: it will develop into an R7 photoreceptor if it receives appropriate instructions, or it will adopt a nonneuronal cone cell fate if these instructions are disrupted by mutations in *sev* or *boss*.

Lack of the *sev* or *boss* functions might be expected to block R7 development, either because the inducing cells are unable to provide a signal or because the receiving cells are unable to perceive or implement the signal. It has been possible to distinguish between these alternatives by determining which cells require a wild-type allele of the *sev* or *boss* genes for development to proceed normally. Mosaic individuals in which some somatic cells have lost their wild-type copy of a gene of interest can be generated through mitotic recombination (Figure 4). In this way, it has been demonstrated that the *sev* gene product is required only in the presumptive R7 cell itself (Campos-Ortega et al 1979, Tomlinson & Ready 1987a), implying that the gene has a role in receiving or implementing a signal required for R7 cell development. In

of coordinated cell movements. Some cells in the initial cluster dissociate, giving rise to clusters containing six or seven cells. (*c*) The five-cell precluster contains the cells that will give rise to R2, R3, R4, R5, and R8. (*d*) The cells that are not part of the five-cell precluster undergo an additional round of cell division (m). (*e*) The R1 and R6 cells then join the cluster. (*f*) They are followed by R7. When the nuclei of R1 and R6 rise into an apical location, the nucleus of the R7 precursor is found basally. The nucleus of the R7 precursor rises apically. As it begins to differentiate, its nucleus shifts more basally. (*g*) The cone cells then surround this core of neuronal cells. The upper panel shows a cross-sectional view of the developing cluster at the apical region of the disc epithelium. The lower panel shows the profile of these cells along the apical-basal axis. A single bundle of axons extends from the basal region of each cluster of photoreceptor neurons. Although the nuclei of the developing cells move up and down in the disc epithelium, there is very little lateral movement of cells.

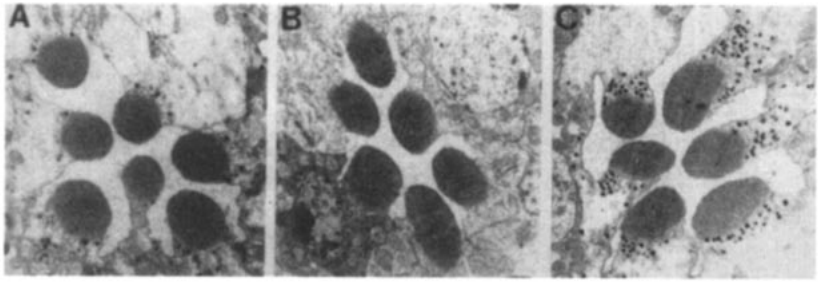

Figure 3 The R7 cell is missing in both *sev* and *boss* mutant ommatidia. (*A*) Wild type: In a distal section of an ommatidium the cellular profiles of seven of the eight R cells are seen. The rhabdomere of the R7 cell in the center of the ommatidium is smaller than the rhabdomeres of the surrounding R1–R6 cells. The R7 neuron has a unique projection pattern, cellular morphology, and spectral sensitivity. The R7 cell is missing in both *sev* (panel *B*) and *boss* mutants (panel *C*). Scale bar, 1 μm. From Reinke & Zipursky 1988; reprinted by permission of publisher.

contrast, *boss* is required in, and only in, the R8 cell for successful development of the R7 cell in the same ommatidium (Reinke & Zipursky 1988). These results indicate that *boss* is required to generate a product in R8 that is necessary, not for the development of R8, but for the development of the R7 cell that it contacts. As described below, the structures and biochemical activities of the Sev and Boss proteins fit their inferred roles quite well.

SEVENLESS IS A RECEPTOR PROTEIN TYROSINE KINASE

The sequence of the *sevenless* gene revealed a striking similarity to the primary sequence of receptor tyrosine kinases (RTK) (Hafen et al 1987). Biochemical studies demonstrated that (Sev) is synthesized as a 280-kDa glycoprotein precursor that is subsequently cleaved into two subunits of 220 kDa (N-terminal) and 58 kDa (C-terminal) that remain associated by noncovalent interactions (Simon et al 1989). Sev differs from other RTKs in its unusually large extracellular domain (~ 2000 amino acids) and in the noncovalent nature of the association between its subunits. Gel filtration experiments suggest that Sev may exist as an α_2 β_2 heterotetramer. The carboxy-terminal subunit contains the transmembrane domain and a cytoplasmic domain that is highly homologous to known tyrosine kinases. In addition, a lower level of homology extends into the extracellular domain with the vertebrate RTK, the c-ros protein (Chen et al 1991a; Narayana & Nagarajan 1992); however, it is unclear if there is any functional homology between these two receptors. The Sev protein has been shown to have protein tyrosine kinase activity in vitro (Simon

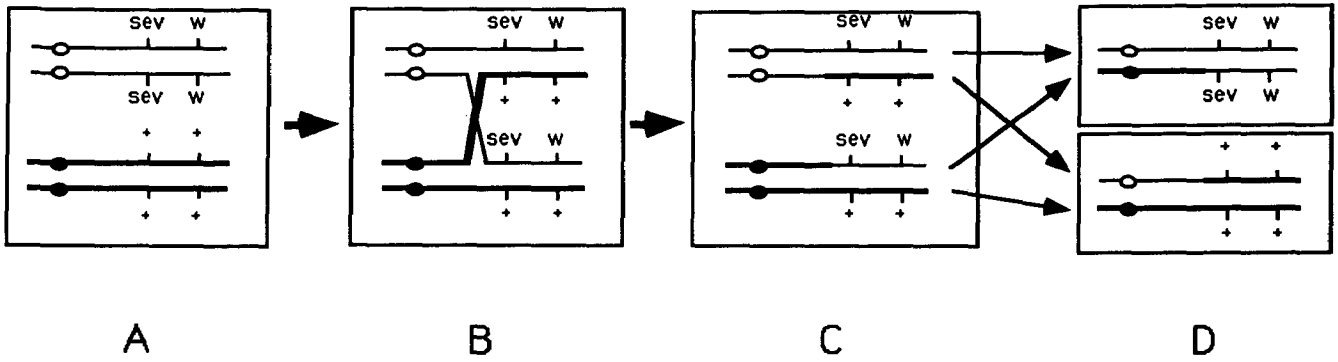

Figure 4 Genetic mosaic analysis of *sev* function. Genetic mosaic analysis provides a way to determine the genetic requirement for a specific gene in any of the pigmented cells in the eye. In this example we describe the method used to determine the cellular requirement for the *sev* gene. The goal of this experiment is to induce clones of mutant cells in an otherwise wild-type eye. Owing to the nonclonal mechanism of ommatidial assembly, ommatidia containing both mutant and wild-type cells will be found. The genotype of each cell at the *sev* locus can be assessed by determining the presence of pigment granules controlled by the white (*w*) gene: pigmented cells carry the wild-type allele of *sev*, and conversely unpigmented cells are homozygous for the mutant *sev* allele. The cellular requirement for *sev* can be determined by correlating the phenotype of each ommatidium (i.e. the presence of the R7 cell) and the genotype of each cell. Genetic mosaic analysis of *sev* showed that it was strictly required in R7; no unpigmented R7 cells were found. In contrast, a similar genetic mosaic study with the *boss* gene showed that the R7 cell could form regardless of its *boss* genotype. An examination of the genotype of other cells in mosaic ommatidia showed that the presence of an R7 cell was strictly dependent on the *boss* genotype of the R8 cell. In the example above, females heterozygous for both *sev* and *w* are subjected to X rays in late first instar of larval development. X rays stimulate mitotic recombination. Mitotic recombination occurs subsequent to chromosomal duplication (*A*, *B*, and *C*). Mitotic recombination between *sev* and the centromere can give rise to two daughter cells that are genotypically distinct (*D*). The daughter cell that is homozygous for the mutant alleles of both *sev* and *w* will give rise to an unpigmented patch of tissue in the adult eye. Its sister cell will give rise to a red patch of tissue that is indistinguishable from the heterozygous parental cell. In practice, only a fraction of the irradiated larvae give rise to mosaic eyes. Not surprisingly, an eye rarely contains more than one patch.

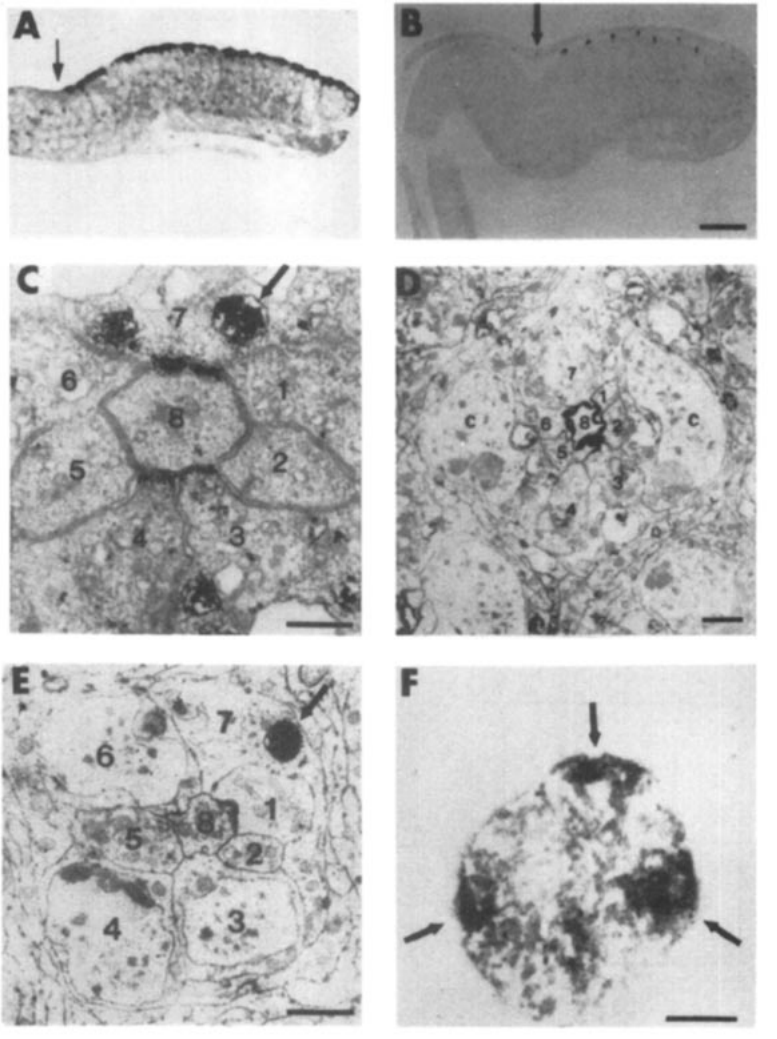

Figure 5 Immunolocalization of Sev and Boss in the developing eye imaginal disc. (*A*) and (*B*) The distribution of Sev and Boss proteins, respectively, along the apical basal axis in the eye imaginal discs. The arrows indicate the position of the morphogenetic furrow. Scale bar, 25 μm.

(*C*) Electron micrograph of a developing cluster stained with an antibody to Sev showing expression in R3, R4, and R7. Sev is also expressed in R1 and R6 and in cone cell precursors that surround the R cell cluster and cells between clusters (not shown in this micrograph). In this micrograph the Sev protein is seen capping on the R8 cell. In addition, Sev immunoreactivity is seen in a multivesicular body (MVB) in the R7 cell (*arrow*). Scale bar, 0.5 μm.

(*D*) Expression of Boss on the surface of the R8 cell. Scale bar, 1 μm.

(*E*) Accumulation of Boss immunoreactivity in an MVB in R7. Various lines of evidence indicate that the accumulation of Boss in R7 is the result of its transfer from R8 via a Sev-receptor mediated process. Scale bar, 1 μm.

et al 1989), and this activity appears to be essential for Sev function in vivo (Basler & Hafen 1988).

Expression of Sevenless

Sev is expressed in each developing ommatidium prior to differentiation of the R7 cell and is found transiently at high levels in at least nine cells (Banerjee et al 1987, Tomlinson et al 1987). In all Sev-expressing cells, the heaviest staining occurred in the microvilli at the apical surface of the epithelium (Figure 5*A*). In addition, staining was found in large multivesicular bodies (MVBs) in all cells in which Sev was detected at the cell surface (Tomlinson et al 1987). A striking staining of plasma membranes occurred at the level of the adherens-type junctions in the first few microns of the tissue below the microvilli. Here, accumulation of stain within R3, R4, and R7 was seen precisely at the position where they oppose R8, but not where they abut other cells or below the level of the adherens junctions (Figure 5*C*). This led Tomlinson et al (1987) to propose that the ligand for the Sev RTK was expressed on the surface of the R8 cell.

Expression of Sev is first detected prior to the cell division that generates the R7 precursor cell. The onset of expression in R3, R4, and other cells of the ommatidium closely follows the sequence of, but precedes, the maturation of these cells, as revealed by their expression of neural antigens (Tomlinson & Ready 1987b). In particular, Sev staining is seen at high levels in the R7 precursor cell some eight hours prior to overt differentiation (Tomlinson et al 1987). However, unlike the staining revealed with antibodies to neuronal markers, the expression of Sev is transient, and only some photoreceptors express detectable levels, with R2, R5, and R8 being the exceptions (Tomlinson et al 1987). This complex pattern of expression appears to be controlled strictly at the level of transcription (Basler et al 1989; Bowtell et al 1989a, 1991).

The appearance of Sev prior to the specification of R7 and its presence in many cells are consistent with a role for Sev in receiving a signal required to induce the R7 developmental pathway. The observation that the Sev expression pattern was much more complex and dynamic than those observed for all other RTKs suggested that its distribution might be important in determining which cells respond to the proposed inductive signal. However, this specificity was not compromised when Sev was expressed in all cells of the eye disc under the control of a heat-shock promoter (Basler & Hafen 1989,

(*F*) High magnification of view of an MVB in R7 stained with the Boss antibody. Scale bar, 0.3 μm.

Panels *A* and *C* are from Tomlinson et al (1987); reprinted by permission of publisher. Panels *B* and *D–F* are from Krämer et al (1991); reprinted by permission of publisher.

Bowtell et al 1989b). Thus, the complex spatial distribution of Sev is not a crucial part of the positional information specifying R7 cell fate. Consistent with a role in receiving a transient inductive signal, expression of Sev was found to be required only during a brief period of ommatidial development; Sev activity is required for the initiation of R7 cell development but not for its subsequent differentiation, maintenance, or function (Basler & Hafen 1989, Bowtell et al 1989b). Experiments with temperature-sensitive alleles of Sev indicate that the Sev-mediated signal must be maintained for several hours for R7 induction to occur (Mullins & Rubin 1991).

BRIDE OF SEVENLESS IS A LIGAND FOR THE SEV RTK

Boss is a Multiple Membrane Spanning Protein

The requirement of *boss* in the R8 cell led to the parsimonious model that *boss* encodes the inductive ligand for Sev (Reinke & Zipursky 1988). As a first step toward testing this model, *boss* was cloned and its primary structure was deduced from the cDNA sequence (Hart et al 1990). The *boss* gene encodes a polypeptide of 896 amino acids. Hydropathy analysis led to the prediction that Boss is a transmembrane protein with an extracellular N-terminal domain of 498 amino acids, 7 segments of sufficient length and hydropathy to span the membrane, and a C-terminal cytoplasmic tail of 115 amino acids. Biochemical and immunohistological studies confirmed that the N- and C-termini are oriented toward the outside and inside of the cell, respectively (Cagan et al 1992). Although the topology of Boss is remarkably similar to that of the superfamily of G protein-linked receptors, there is no primary sequence homology.

Boss is Expressed in the Apical Region of the R8 Cell

Boss was shown to be expressed at a time and place consistent with its proposed role as the R7 inductive ligand. As described above, Sev was expressed in the apical-most region of the R7 precursor cell, as well as other cells in the disc, and was required in a precisely defined window of developmental time. Immunohistology at the light and electron microscope level also showed that Boss was localized to the most apical region of the disc, in this case specifically on the membranes of the R8 cell (Figure 5*B, D;* Krämer et al 1991). Expression of Boss commences prior to R7 induction and remains in the R8 cell throughout the period required for *sev* activity, as defined with heat-shock promoter-driven constructs (hs sev; Basler & Hafen 1989, Bowtell et al 1989b; see below) and temperature-sensitive alleles of *sev* (Mullins & Rubin 1991).

Although Boss is also expressed in a large number of developing sensory

neurons, it does not appear to be required for their development or the development of associated sensory structures. In principle, Boss could play a more subtle developmental role or a physiological role, or its expression in these cells may simply be gratuitous. It is important to note, however, that Boss and Sev are not coexpressed in tissues outside of the developing eye; apparently the Boss-Sev pathway is exquisitely specific for regulating the development of only one cell type in the entire organism.

Evidence for Interaction between Boss and Sev from In Vitro Studies

A tissue culture approach was used to test whether Boss and Sev directly interact (Krämer et al 1991). Two derivatives of the *D. melanogaster* S2 cell line were constructed, one expressing Sev (SL2-sev) (Simon et al 1989) and the other expressing Boss (S2:boss). These two cell lines formed single cell suspensions when incubated alone but aggregated upon mixing. Aggregates contained approximately equal numbers of S2:boss and SL2-sev cells. Aggregation was inhibited by antibodies to Boss and Sev and was calcium-dependent.

The ability of Boss to stimulate Sev tyrosine kinase activity was shown in two ways (Hart et al 1993). First, incubation of S2:boss cells with SL2-sev cells led to a rapid and transient increase in tyrosine phosphorylation on Sev, as assessed on protein immunoblots probed with antibodies to phosphotyrosine. Similar results were obtained with membrane preparations from S2:boss cells. An increase in tyrosine phosphorylation was not seen when an S2 cell line expressing a catalytically inactive Sev mutant was incubated with S2:boss cells. Histological studies in culture provided additional evidence that Sev was locally activated at the site of interaction between the S2:boss and the SL2-sev cells. Aggregates of S2:boss and SL2-sev cells were triple-stained with antibodies to Sev, Boss, and phosphotyrosine. The Sev protein and phosphotyrosine immunoreactivity were concentrated at the site of contact between the two cells; there was no apparent clustering of Boss. In contrast, although the catalytically inactive Sev protein capped on the Boss-expressing cell, a local increase in tyrosine phosphorylation was not observed. These data indicate that the increase in tyrosine phosphorylation likely results from activation of the Sev tyrosine kinase itself.

Internalization of Boss into the R7 Precursor Cell

The studies described in the previous sections indicate a direct interaction between Boss and Sev in vitro. Does this happen in vivo? Detailed histological studies provided evidence for such an interaction. By using immuno-electronmicroscopy, Krämer et al (1991) have found Boss within a multivesicular

body (MVB) in the R7 precursor cell (Figure 5*E, F*). This appears to be the same structure in which Sev immunoreactivity was also detected, as described earlier in this review. MVBs are morphologically similar to late endosomal compartments in mammalian cells within which internalized receptor-ligand complexes dissociate; the ligands are then sorted to the lysosome for degradation, and the receptor, depending on the specific receptor in question, is either recycled to the cell surface or degraded.

Several lines of evidence indicated that Boss was transferred from the R8 cell to the R7 precursor cell through its interaction with Sev. First, Boss immunoreactivity was observed on the rough endoplasmic reticulum (RER) of the R8 cell, but not on the RER of any other cells in the developing eye disc. Hence, Boss is only translated in R8. Second, internalization of Boss by the R7 cell was strictly dependent upon Sev. Internalization of Boss is not sufficient for R7 development; an R7 precursor cell, expressing a kinase-negative Sev mutant protein that binds Boss in vitro, internalizes Boss in vivo even though the cell is transformed into a cone cell. Moreover, internalization is not a specialized property of the R7 precursor cell, as it is observed in aggregates of S2:boss and SL2-sev cells.

To assess which regions of Boss were internalized into Sev-expressing cells both in culture and in the developing eye disc, the Boss distribution was determined in studies that used antibodies to several different extracellular and intracellular epitopes. These studies showed that the entire Boss protein was internalized into the SL2-sev cells. Although the mechanism by which this occurs is not known, it seems likely that it proceeds by a variation of receptor-mediated endocytosis recently described in Aplysia; Bailey et al (1992) showed that double-membrane clathrin-coated pits and double-membrane clathrin-coated vesicles formed during active periods of membrane remodeling of cell contacts in culture. Interestingly, internalization of Boss requires the *shibire* (*shi*) gene product, a homologue of rat dynamin (Chen et al 1991b, Krämer et al 1991, Van der Bliek & Meyerowitz 1991). In temperature-sensitive alleles of *shi* incubated at nonpermissive temperatures, endocytic intermediates accumulate in which deep invaginations of the plasma membrane form, but vesicles fail to pinch off (Kosaka & Ikeda 1983, Masur et al 1990). In addition, very brief incubation at the nonpermissive temperature results in marked loss of clathrin-coated vesicles and pits.

Although Boss internalization provides strong evidence for a direct interaction between Boss and Sev in vivo, internalization does not appear to be an obligatory requirement for R7 induction. A constitutively activated form of Sev (Basler et al 1991; see below) promotes Boss-independent R7 development. It remains possible, however, that internalization of an activated receptor is a necessary requirement for induction and that during normal development the receptor is only active when complexed to Boss.

The Seven Transmembrane Domains of Boss are Required for its Function

Although other transmembrane ligands have been identified (e.g. Steel Factor; see Witte 1990), Boss is unique in containing seven putative transmembrane segments. Is the seven-transmembrane region of Boss important for its function? Several mutant forms of Boss were tested for their function both in vivo and in vitro (Hart et al 1993). The extracellular domain (designated EXboss) comprising 480 amino acids was purified to apparent homogeneity by lectin-affinity chromatography. Radiolabeled EXboss did not bind with high affinity to Sev. However, it did inhibit aggregation of S2:boss with the SL2-sev cells and Boss activation of the Sev tyrosine kinase. Expression of EXboss in the developing eye disc was shown to inhibit R7 development in a genetically sensitized system in which the expression of wild-type Boss was limiting. The inability to function as an agonist did not result from a requirement for membrane anchoring, as two transmembrane forms, one containing TM 7 and the other TM 1, 6, and 7, functioned neither as agonists nor antagonists of Sev. These data suggest that the extracellular domain has a low affinity for Sev and that the region containing the transmembrane segments is required for Boss function. The transmembrane domain may be required for the correct folding of the extracellular domain, or it may interact directly with Sev. Given the topological similarity of Boss to members of the seven-transmembrane superfamily of G-protein linked receptors, it is intriguing to consider the notion that Sev activates a G-protein cascade in the R8 cell. If such a cascade is activated, it is unclear what role it would play; development of the R8 cell is normal in *sev*, *boss*, or a *sev;boss* double mutant background.

A GENETIC APPROACH TO DISSECTING AN RTK SIGNAL TRANSDUCTION CASCADE

Activation of the Sev tyrosine kinase must result in intracellular changes in the R7 precursor cell that cause it to adopt the R7 cell fate rather than that of a cone cell. The mechanisms by which RTKs effect changes in cell physiology are still poorly understood. Biochemical studies with mammalian RTKs have led to the identification of proteins that bind to or are phosphorylated by them (see Cantley et al 1991). Although some of these interactions suggest potential mechanisms of signal transmission, their role in vivo is still unclear.

The Sev pathway is ideal for genetic approaches to understanding signaling by RTKs. First, both the R7 cell and the Sev protein are dispensable for viability and fertility. Second, the functioning of this signaling pathway can

be inferred from the presence of the R7 cell in a live, anesthetized fly. Screens for mutations that alter the strength of Sev signaling can be used to identify other elements in the pathway.

Simon et al (1991) carried out a systematic genetic screen for dominant enhancers of *sev* that would presumably decrease the effectiveness of signaling by Sev. By adjusting the temperature at which flies carrying a temperature-sensitive allele of *sev* were grown, it was possible to adjust Sev activity to a level barely above the threshold necessary for R7 cell formation. Under these conditions, some 80% of the ommatidia had an R7 cell. Small reductions in the abundance or activity of other elements of the pathway might then be expected to lower signal strength sufficiently to cause a *sev* phenotype in most of the ommatidia. This sensitivity allowed Simon et al (1991) to identify seven genes encoding putative downstream elements of the pathway, by screening for genes in which inactivation of only one copy of the gene—which would be expected to reduce the level of gene product by half—resulted in the absence of the R7 cell. As the other copy of the gene remained functional, they were able to identify these loci even though their functions were essential for viability.

To determine whether these genes defined components of the Sev pathway specifically or RTK systems more generally, Simon et al (1991) assessed the ability of these mutations to suppress the *Ellipse* (Elp) mutation (Baker & Rubin 1989, 1992), a dominant allele of the *D. melanogaster* EGF receptor. The *Elp* mutation gives rise to a disorganized eye with a reduced number of ommatidia. Four *sev* enhancers were suppressors of *Elp*, suggesting that they encode proteins that participate in the transduction of signals from at least two different RTKs. One of the four loci corresponds to the *Ras1* gene, which encodes a p21*ras* protein (Simon et al 1991, Neumann-Silberberg et al 1984) and is the *D. melanogaster* homologue of human H-ras, Ki-ras, and N-ras. Studies in vertebrates also implicate ras in early steps in signaling cascades downstream from RTKs (Smith et al 1986).

Activation of Ras1 is a Key Consequence of Sev Activation

To determine whether Ras1 activation alone is sufficient for Sev-mediated signaling, Fortini et al (1992) used *sev* gene regulatory sequences to express dominant-activating Ras1 alleles in those cells of the eye imaginal disc that normally express Sev; the dominantly active Ras1 mutations were constructed in vitro based on studies of mammalian Ras proteins (Bourne et al 1990a,b). Previous studies by Basler et al (1991) demonstrated that expression of a truncated, constitutively active Sev protein under Sev gene control results in *boss*-independent rescue of the normal R7 cell and transformation of cone cell precursors into supernumerary R7 cells (Basler et al 1991; see below). Identical results were obtained with activated Ras1 ($Ras1^{val12}$), but not

wild-type Ras, indicating that Ras1 activation can substitute for all of the Sev-mediated signal (Fortini et al 1992). However, these results cannot exclude the possibility that endogenous Ras1 protein is not activated to the levels achieved by $Ras1^{val12}$ or that normal Sev-mediated signaling requires additional pathways operating in parallel to the Ras1 pathway.

Suppression of the *sev* phenotype by dominant activated $ras1^{val12}$ is strikingly similar to genetic interactions in *Caenorhabditis elegans* vulval development. Vulva formation requires *ras* (reviewed in Sternberg & Horvitz 1991). Dominant activating mutations in the Ras protein bypass the requirement for receptor and produce a multivulva phenotype. The central role of Ras activation in signaling by these two structurally dissimilar RTKs is surprising, as mammalian RTKs are thought to act on a variety of downstream targets. Moreover, Ras activation alone cannot explain how different physiological responses are elicited in a single cell type by stimulation of different RTKs (Ullrich & Schlessinger 1990, Cross & Dexter 1991; see below). Nevertheless, the genetic results obtained in *D. melanogaster* and *C. elegans* suggest that Ras activation may be a primary means by which RTKs exert their effects in many signal transduction pathways.

How is Ras1 Activated?

How does the activation of Sev result in the activation of Ras1? An outline of the answer is emerging from the analysis of the other mutations isolated in genetic screens, such as those described above. The activity of Ras proteins is regulated by bound guanine nucleotides (reviewed by Bourne et al 1990a,b); the GTP-bound state is active, whereas the GDP-bound state is inactive. The ratio of GTP:Ras to GDP:Ras is determined by two antagonistic reactions (see Figure 6). An active GTP:Ras molecule is inactivated by the intrinsic GTPase activity of the Ras protein, a process that is greatly stimulated by RasGAP. An inactive GDP:Ras molecule is activated by the exchange of the bound GDP molecule for a GTP molecule, a reaction that is stimulated by guanine nucleotide exchange proteins (GNEPs).

The product of the *Son of sevenless* (*Sos*) locus is required for R7 development. Loss of function mutations at the *Sos* locus were isolated in the Simon et al (1991) screen, and a gain-of-function mutation of *Sos* was identified as a suppressor of a *sev* allele that retained a low level of activity (Rogge et al 1991). The Sos protein shows sequence similarity to the *Saccharomyces cerevisiae* CDC25 protein, a known GNEP (Simon et al 1991, Bonfini et al 1992), providing further support for a model in which the stimulation of Ras activity is a key element in signaling by Sev. Furthermore, it suggests that Sev stimulation may be achieved by activating the exchange of GDP for GTP by Ras proteins. In this context, the activity of another protein encoded by the E(sev)2B gene originally identified in the Simon et al

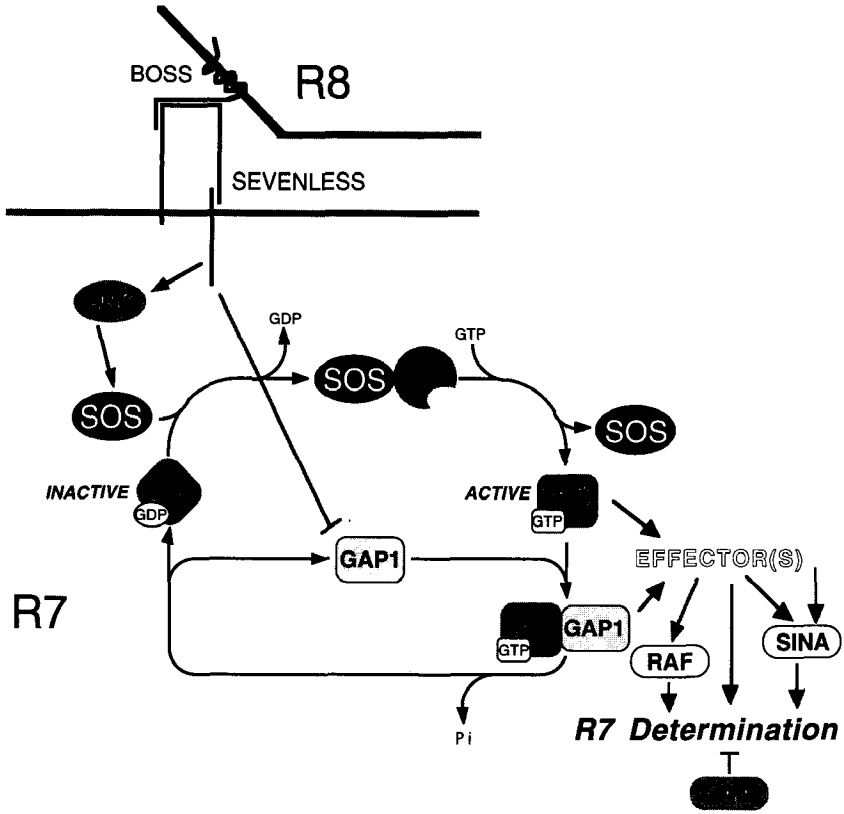

Figure 6 Model for the signal transduction pathway that acts downstream of Sevenless. See text for details.

(1991) screen is intriguing. The gene encodes a protein of the structure SH3-SH2-SH3 (Olivier et al 1993, Simon et al 1993), similar to the *C. elegans* SEM 5 (Clark et al 1992) and human GRB2 proteins (Lowenstein et al 1992). This gene, which has been renamed *downstream of receptor kinases* (*drk*), encodes a protein (Drk) that is required for activation of $p21^{Ras1}$, but not for any subsequent event, and it can bind both to Sev and to Sos (Olivier et al 1993, Simon et al 1993). These results suggest that Drk may stimulate Sos to activate $p21^{Ras1}$ by linking Sev and Sos in a signaling complex.

A RasGAP, Gap1, that regulates Ras1 during eye development has also been identified. Gap1 encodes a protein similar to mammalian RasGAP (Gaul et al 1992). Loss-of-function mutations of Gap1 mimic constitutive activation of Ras, implicating Gap1 as a negative regulator of Ras1 and hence R7 determination (Buckles et al 1992, Gaul et al 1992, Rogge et al 1992). Gap1 presumably functions by inhibiting *D. melanogaster* Ras1 protein; signaling

by Sev overcomes this inhibition. The available data show that the levels of both the Sos and Gap1 activities can be limiting steps in the decision by the R7 precursor cell to become an R7 cell. Inactivation of one copy of the Sos gene decreases the effectiveness of Sev signaling (Simon et al 1991), whereas inactivation of one copy of the Gap1 gene increases it (Gaul et al 1992). Although these results are consistent with the possibility that Sev regulates the activity of both Sos and Gap1, biochemical studies will be required to determine whether these proteins are directly regulated by Sev.

The expression of Gap1 is highly restricted, whereas that of Ras1 is not. For example, within the eye disc, Gap1 expression is found only in the region posterior to the morphogenetic furrow, but Ras1 expression is found throughout the entire disc (Segal & Shilo 1986). Furthermore, whereas *Ras1* and *Sos* loss-of-function mutations are organismal lethal, *Gap1* homozygotes are viable. This suggests that distinct GTPase activating proteins may regulate Ras1 in different developmental pathways.

Raf May Function Downstream from Ras1 in R7 Determination

Little is known about the molecular mechanisms downstream from ras. Several lines of evidence in vertebrate systems indicate that the product of the c-raf proto-oncogene functions downstream of Ras in at least some cells (Kolch et al 1991, Wood et al 1992). Studies of the Torso RTK system in the *D. melanogaster* embryo place the *D. melanogaster* raf homologue, the product of the *l(1)polehole* gene (simply referred to here as the *raf* locus), downstream of the Torso RTK (Ambrosio et al 1988). Does *raf* function in the Sev RTK system downstream of *ras*? Dickson et al (1992b) have recently addressed this question. They showed that although complete loss-of-function mutations of *raf* are recessive lethals, hemizygous males carrying a weak *raf* allele (raf^{HM7}) do survive into adulthood. Sections of raf^{HM7} eyes reveal that many of the ommatidia lack R7 cells; other R cells are also occasionally missing. Conversely, as seen with Sev and Ras, a dominantly activated form of Raf also leads to additional R7 cells. Activation of Raf was achieved by fusing the extracellular and transmembrane domains of a constitutively active torso RTK mutant to the Raf kinase domain.

Two lines of evidence suggest that raf may act downstream of ras. First, the massive increase in R7 cells that develop as a consequence of expressing the dominantly-activated Ras protein is reverted in a genetic background hemizygous for the raf^{HM7} allele. Second, reducing the gene dosage of *ras*, which as we saw earlier enhances the phenotype of a hypomorphic *sev* allele, has no effect on the development on the phenotype of a dominant *raf* mutation. Genetic analysis in *C. elegans* has also placed raf downstream from ras in

the signal transduction cascade regulating vulval development (Han et al 1993).

sina Encodes a Nuclear Protein Essential for R7 Development

The *sina* gene encodes a nuclear protein required for R7 determination; loss of *sina* activity has the same effect on the presumptive R7 cell as does loss of *sev* function (Carthew & Rubin 1990). However *sina*, unlike *sev*, also functions in other tissues and other cells in the eye disc. The precise role of *sina* within the R7 precursor cell remains unclear; however, *sina* appears to act downstream of activated Ras (Fortini et al 1992) and activated Raf (Dickson et al 1992b). Modification of *sina* activity may be one end-point for the signal transduction pathway initiated by Sev, or alternatively, *sina* may function in parallel to it.

MULTIPLE MECHANISMS RESTRICT THE R7 PATHWAY OF DEVELOPMENT TO A SINGLE PRECURSOR CELL

Why does only one of the Sev-expressing cells in the developing ommatidium assume an R7 cell fate? In addition to the R7 precursor cell, Sev is expressed in the precursors to additional R cells (R1, R3, R4, and R6), the so-called mystery cells, the cone cells, and cells between developing clusters (Tomlinson et al 1987). Multiple mechanisms act in concert to restrict induction to the R7 precursor cell, including spatial localization of the ligand, commitment to alternative developmental pathways, and the existence of antagonistic, yet reversable, functions in uncommitted cells.

Spatial Localization of Boss Restricts R7 Induction

During normal development, the Sev-expressing cone cell precursors and cells between clusters do not contact the Boss-expressing R8 cell. To test whether the precise localization of Boss prevents these Sev-expressing cells from becoming R7 cells, Van Vactor et al (1991) examined the effects of expressing Boss in all cells in the eye disc under the control of the hsp 70 promoter. Cone cell precursors assume R7 cell fates in response to ectopic Boss expression (see Figure 7). Hence, during normal development the cone cell precursors fail to assume an R7 cell fate, owing to the spatial localization of the inductive ligand. Very similar results were obtained by expressing a constitutively active Sev RTK under the control of a *sev* enhancer element (Basler et al 1991, Dickson et al 1992a).

The restriction of the cone cell precursors is not exclusively controlled by the spatial localization of Boss. As described above, mutations at the *Gap1* locus lead to *sev*-independent transformation of cone cells into R7 cells

(Buckles et al 1992, Gaul et al 1992, Rogge et al 1992). Hence, during normal development the R7 signaling pathway is constitutively inhibited by Gap1, presumably through its inhibition of Ras. In addition to *Gap1*, the *yan* gene, a putative transcription factor, also represses R7 development, as well as the development of other R cells in the uncommitted cells surrounding early developing ommatidial clusters (Lai & Rubin 1992). These data indicate that both extrinsic and intrinsic mechanisms prevent R7 induction in cells that do not contact R8 (see Figure 8).

Commitment to Alternative Fates Prevents R7 Development in the R1-R6 Precursor Cells

The R1–R6 precursor cells do not assume an R7 cell fate in response to ectopic expression of Boss or in response to activated forms of Sev, Ras, or Raf. Several lines of evidence support the view that these cells are not able to respond because of their commitment to alternative pathways of development. Mutations at the *seven-up* locus, which encodes a member of the steroid receptor superfamily, lead to the transformation of R1, R3, R4, and R6 cells into R7 cells (Mlodzik et al 1990). Some of these cells, but not all, are transformed in a *sev*-dependent fashion. Similarly, mutations in the *rough* gene, which encodes another putative transcription factor containing a homeodomain (Saint et al 1988, Tomlinson et al 1988), also result in ectopic R7 cells (Heberlein et al 1991, Van Vactor et al 1991). Rough is required for the development of R2, R5, and, indirectly, additional R cells in the developing ommatidium (Tomlinson et al 1988). It is not clear which R1–R6 cells assume an R7 cell fate in a *rough* mutant background. The conclusion that additional R7 cells develop at the expense of R1–R6 cells is based largely on the observation of an increase in the number of R7 cells per ommatidium in the adult and a concomitant reduction in the number of R1–R6 cells.

Interestingly, the inability of the Sev-expressing R1–R6 precursor cells to respond to the inductive cue is correlated with the observation that, in contrast to the R7 precursor cell, these cells fail to internalize Boss. Under conditions in which these cells remain competent to respond to the inductive cue (e.g. in a *rough* mutant background), multiple cells, presumably precursors to the R1–R6 cell population, internalize Boss (Van Vactor et al 1991). This suggests that the molecular mechanism restricting the developmental potential of the R7 signaling pathway in the Sev-expressing R1–R6 cells is reflected at the level of the interaction between Sev and Boss; either Boss and Sev fail to bind to each other or the complex cannot be internalized. However, given that constitutively active Sev, Ras, and Raf cannot drive these cells to assume an R7 pathway, additional mechanisms must prevent activation of the R7 pathway in these cells.

The Control of R7 Identity

What is the nature of the Boss inductive cue? Is the R7 precursor cell multipotent, with one signal promoting R7 development, another cone cell development, and yet another R1–R6 development? Or is the R7 precursor cell already considerably restricted in its developmental potential, such that Boss activates an R7 cell fate, whereas in the absence of a signal the cell assumes a default pathway of a nonneuronal cone cell?

This has been a very complex question to address. Ideally one would like to assess the developmental consequences of transplanting the R7 precursor cell to ectopic locations in the developing disc or to regions of the disc representing different developmental times. These manipulations are not feasible. Evidence that the R7 precursor may be different from other cells in the developing disc, prior to the action of *sev*, is the finding that the H214 enhancer trap line is expressed specifically in the R7 precursor even in the absence of *sev* function (Mlodzik et al 1992). The information content of the developmental signal has been inferred from studies with transgenic animals. For instance, the results of ectopic expression of the *rough* gene in the R7 precursor cell suggest a rather nonspecific signal generated by Boss activation of Sev. Expression of *rough* in the R7 precursor cell leads to its transformation into an R1–R6-like cell in a *sev*-dependent fashion (Basler et al 1990, Kimmel et al 1990). This suggests that either *rough* alters the developmental potential prior to activation of Sev or, alternatively, the Rough protein itself is activated by the Sev signal transduction pathway to implement an R1–R6-like pathway of development. Given that Ras and Raf appear to function downstream of the *D. melanogaster* EGF, Torso, and Sev RTKs, and that activated forms of Ras and Raf drive *sev*-independent R7 development, it seems likely that the Sev signal is simply the last step in the sequential limitation of the developmental potential of the R7 precursor.

PERSPECTIVE

The Sev and Boss proteins comprise an unusual receptor/ligand pair. Both proteins have highly restricted distributions and functions, and activation of Sev apparently requires direct contact with Boss-expressing cells. It remains to be seen how many such specialized signaling systems are used during the development of complex organisms. The membrane-bound nature of the ligand makes it much more difficult to discover such signaling systems by using traditional biochemical approaches.

Recent genetic studies highlight the complementary nature of the information that has been, and can be, provided by molecular and genetic approaches to cell signaling. Screens for mutations have provided a way to identify genes

that play an important role in eye development, and analysis of genetic mosaics has been used to determine in what cell the ommatidium requires a particular gene. In combination with detailed analyses of mutant phenotypes, these data indicate whether a gene product is involved in the sending or receiving of signals. For example, *sev* was unequivocally demonstrated to be required only in the R7 cell. Knowledge of the pattern of *sev* gene expression is strikingly uninformative in this regard; Sev is expressed in many cells, not just R7. On the other hand, the genetic analyses do not provide information on the nature of the gene product or its biochemical function. The genetic analyses are equally consistent with *sev*'s encoding a transcription factor, a receptor, or a component of the intracellular signaling machinery. Distinguishing among such alternatives has been the purview of molecular and biochemical studies.

Finally, we note the truly striking degree of evolutionary conservation seen in the components of the Sev-mediated signaling pathway. For example, the Sev tyrosine kinase domain is 47% identical to that of c-ros (Hafen et al 1987), *D. melanogaster* Ras1 is 77% identical to human H-ras (Simon et al

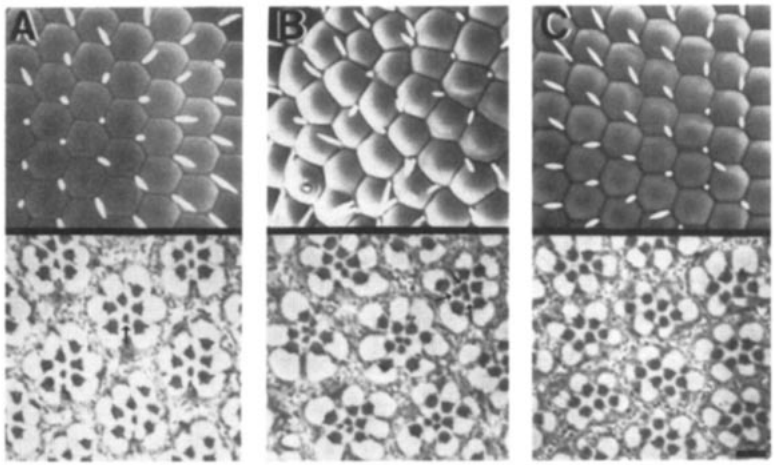

Figure 7 Ectopic activation of the Sev signal transduction pathway leads to the formation of additional R7 cells. Ectopic activation of the R7 pathway has been achieved by ubiquitous expression of Boss or by expression of activated forms of Sev, Ras, or Raf under the control of the *sev* enhancer (i.e. driving expression in all those cells expressing the Sev protein) or the hsp 70 promoter. In each case, additional R7 cells develop from a pool of competent cells (see text). Panel *A* shows a wild-type eye. The results of ubiquitous expression of Boss in a wild-type and *sev* mutant background are shown in panels *B* and *C*, respectively. Note the multiple R7 cells in each ommatidium (*arrow*) in panel *B* and the suppression of this effect in a *sev* mutant background. The upper part of each panel shows a scanning electron micrograph of the surface of the compound eye, and the lower panel shows a light micrograph of a plastic section of an eye of the same genotype. Scale bar in *C* is 5 μm.

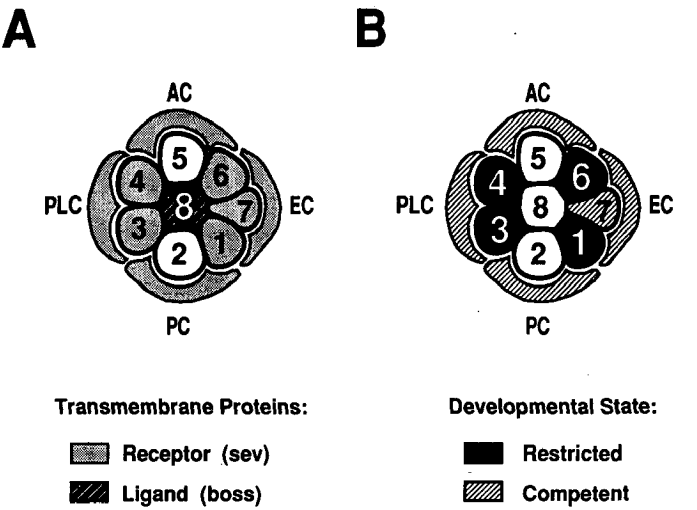

Figure 8 The restriction of the Boss inductive cue to a subset of cells in the developing ommatidium. (*A*) Protein expression in the developing ommatidium. (*B*) The developmental state of cells in the ommatidium. The cone cell precursors and R7 cells are competent to respond to the Boss inductive cue or constitutive activation of Sev, Raf, or Ras. In contrast, the R1, R3, R4, and R6 cells do not assume an R7 cell fate in response to constitutive activation of the R7 pathway, presumably owing to their commitment to alternative fates. Reprinted from Van Vactor et al (1991); reprinted by permission of publisher.

1991), Sos is 45% identical to its mouse homologue (Bowtell et al 1992), drk is 64% identical to the human GRB2 protein (Lowenstein et al 1992), and sina is 83% identical to its mouse counterpart (D. Bowtell, personal communication). This high degree of sequence conservation implies that the function of these proteins has changed little in the 500 million years that separate flies and man. In conclusion, we believe that the experimental advantages of being able to examine mutant phenotype at single cell resolution in a developing tissue that is dispensable for viability and fertility will ensure that new insights into the mechanisms used by neurons to acquire specific identities will continue to emerge from studies of the *D. melanogaster* eye.

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